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SHORT CONTRIBUTION

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Xylose utilisation by recombinant strains of *Saccharomyces cerevisiae* on different carbon sources

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Abstract Autoselective xylose-utilising strains of *Saccharomyces cerevisiae* expressing the xylose reductase (*XYL1*) and xylitol dehydrogenase (*XYL2*) genes of *Pichia stipitis* were constructed by replacing the chromosomal *FUR1* gene with a disrupted *fur1::LEU2* allele. Anaerobic fermentations with 80 g l^{-1} D-xylose as substrate showed a twofold higher consumption of xylose in complex medium compared to defined medium. The xylose consumption rate increased a further threefold when 20 g l^{-1} D-glucose or raffinose was used as co-substrate together with 50 g l^{-1} D-xylose. Xylose consumption was higher with raffinose as co-substrate than with glucose (85% versus 71%, respectively) after 82 h fermentations. A high initial ethanol concentration and moderate levels of glycerol and acetic acid accompanied glucose as co-substrate, whereas the ethanol concentration gradually increased with raffinose as co-substrate with no glycerol and much less acetic acid formation.

Introduction

Xylose is the major pentose found in lignocellulosic materials and after glucose the most abundant sugar available in nature. Naturally xylose-fermenting bacteria, yeast and filamentous fungi have been identified (Jeffries 1990; Hahn-Hägerdal et al. 1993), however, they are less suitable for industrial ethanol production because of low ethanol tolerance, low inhibitor tolerance, and the need for controlled oxygenation during fermentation. Baker's yeast (*Saccharomyces cerevisiae*), a GRAS micro-organism used in wine making, brewing and baking processes, is still the micro-organism of choice for the production of industrial ethanol from fermentable sugars. However, *S. cerevisiae* cannot utilise xylose, but can convert its isomer, xylulose, to ethanol under oxygen-limited growth conditions (Wang and Schneider 1980; Chiang et al. 1981; Senac and Hahn-Hägerdal 1990; Yu et al. 1995). Towards this end, recombinant laboratory strains of *S. cerevisiae* that express the xylose reductase (*XYL1*) and xylitol dehydrogenase (*XYL2*) genes of *Pichia stipitis* under the transcriptional control of the *ADH1* and *PGK1* yeast promoters were constructed (Walfridsson et al. 1997).

In *S. cerevisiae*, several hexose (glucose) transporters have been identified with either a high affinity (Hxt2p, Hxt6p and Hxt7p) or low affinity (Hxt1p, Hxt3p and Hxt4p) for glucose. Snf3p and Rgt2p act as glucose sensors that regulate the expression of *HXT1*, *HXT2*, *HXT4*, *HXT6* and *HXT7* genes in high or low glucose concentrations (Özcan et al. 1996; Boles and Hollenberg 1997). The same transporters presumably also act as transporters for the non-specific uptake of xylose at a much lower affinity constant ($Km = 49–300$ mM) than glucose (1–28 mM), fructose (6–40 mM) or mannose (12–27 mM) (Heredia et al. 1968; Kotyk 1967; Serraro and Delafuente 1974; Busturia and Lagunas 1986; Van Zyl et al. 1993). These hexoses, when used at high concentrations as co-substrate with xylose, saturate the transport system, inhibiting the transport of xylose and thus xylose assimilation (Meinander and Hahn-Hägerdal 1997). *S. cerevisiae*, as found for all other microorganisms, preferentially uses glucose as carbon source; a phenomenon commonly referred to as catabolite (glucose) repression (Gancedo 1998).

The combined effects of transport inhibition, the absence of high affinity transporters and the induction of glucose repression when glucose is used as co-substrate at high concentrations, render glucose a less ideal co-substrate for efficient xylose consumption and assimilation by recombinant *S. cerevisiae* strains. However, raffinose, a trisaccharide composed of a galactose,

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glucose and fructose moiety (O-α-D-galactopyranosyl-[1→6]-α-D-glucopyranosyl-[1→2]-β-D-fructofuranoside), may not present the undesirable side-effects of glucose as co-substrate. Most laboratory strains of *S. cerevisiae* can split off and utilise the fructose moiety of raffinose (with the aid of invertase), however do not have melibiase to degrade the remaining melibiose moiety (Gancedo and Serrano 1989; Gasent-Ramirez et al. 1995). The slow release of fructose by invertase and concomitant transport of this hexose into the yeast cell results in a low effective fructose concentration outside the cell. Therefore, glucose repression is absent and the expression of *HXT2*, *HXT4*, and *HXT6* genes that encode for high affinity glucose transporters is induced (Liang and Gaber 1996; Reifenberger et al. 1997), enhancing xylose uptake. This paper describes the construction of genetically stable autoselective strains of *S. cerevisiae* H158 expressing the *XYL1* and *XYL2* genes from episomal plasmids. Xylose consumption and assimilation were determined in defined and complex media. The effect of glucose versus raffinose as co-substrate on xylose utilisation was also investigated.

Materials and methods

Strains, plasmids, media and cultivation conditions

Table 1 summarises the relevant genotypes and sources of the strains and the plasmids used in this study. Plasmids were amplified in *Escherichia coli* JM101. Bacteria were cultivated at 37 °C in Luria-Bertani medium containing 100 μg/ml ampicillin for plasmid selection (Sambrook et al. 1989). Yeast cells were grown at 30 °C in 100 ml YPD (yeast extract, 10 g l^{-1}; peptone, 20 g l^{-1}; D-glucose, 20 g l^{-1}) and synthetic complete [yeast nitrogen base (Difco, Detriot, USA; supplemented with the required growth factors), 7.2 g l^{-1}; D-glucose, 20 g l^{-1}] media. Solid media contained 20 g l^{-1} agar.

Fermentations were performed essentially as described by Yu et al. (1995) either in complex YP medium (yeast extract, 10 g l^{-1}; peptone, 20 g l^{-1}) or defined medium (Verduyn et al. 1992, supplemented with leucine, 2.5 g l^{-1}; uracil, 0.5 g l^{-1}; tryptophan, 0.5 g l^{-1}; histidine, 0.5 g l^{-1}; aspartate, 0.5 g l^{-1}; glutamate 0.5 g l^{-1}) with D-xylose, D-glucose or raffinose as carbon source. Pre-cultures of *S. cerevisiae* strains were prepared overnight in YP medium at 30 °C. The yeast cells were harvested, washed with sterile 9 g l^{-1} NaCl and fresh medium inoculated to 5 g l^{-1} washed cells. Fermentations in 25-ml plugged bottles filled with 25 ml yeast cultures were performed at 30 °C for up to 82 h with magnetic stirring. During fermentation, the cultures became anaerobic. Samples were withdrawn at regular intervals and stored at −20 °C.

DNA manipulation and transformation

Standard methods for DNA manipulations were employed (Sambrook et al. 1989). Linear DNA fragments were transformed into yeast cells according to the dimethyl sulfoxide method of Hill et al. (1991).

Analytic methods

Xylose, xylulose, glucose, raffinose, xylitol, glycerol, acetate and ethanol concentrations were determined by high-performance liquid chromatography (Varian, California, USA) using an Aminex ion-exclusion HPX-87H cation-exchange column (BioRad, California, USA) at 65 °C with 5 mM H_2SO_4 as the mobile phase. The compounds were detected with a refractive-index detector (410 differential refractometer, Waters, Milford, USA). Samples for substrate/product analysis were filtered through 0.22 μm filters and diluted 20-fold in dH_2O before HPLC injection. The cell concentrations (g l^{-1}) of the different pre-cultures were determined by a microwave oven procedure as described by Yu et al. (1995).

Construction of autoselective *fur1 S. cerevisiae* strains

Autoselective strains of *S. cerevisiae* that would allow plasmid selection in uracil-containing medium, were constructed as devised by Loison et al. (1986). The wild-type *FUR1* gene of *S. cerevisiae* H158[pY6] (Walfridsson et al. 1997) was replaced with a defective *fur1::LEU2* allele, by homologous recombination (Rothstein 1983), using the 3.2-kb *Nco*I–*Nsi*I linear DNA fragment from plasmid pDF1 (La Grange et al. 1996).

Results and discussion

Comparison of xylose utilisation in defined versus complex medium

Xylose utilisation by *S. cerevisiae* strain H158[pY6] (Walfridsson et al. 1997) could not be evaluated in complex medium, but only in synthetic (SC^{-Ura}) medium to ensure plasmid selection. Autoselective xylose-utilising strains of *S. cerevisiae* were therefore constructed by replacing the chromosomal *FUR1* gene with a disrupted *fur1::LEU2* allele. The autoselective xylose-utilising strains of *S. cerevisiae* allowed the comparison of xylose utilisation in defined medium and complex YP medium (Fig. 1). Fermentations of *S. cerevisiae* H158[*fur1*::*LEU2* pY6] were monitored for 76 h in defined and YP medium containing 80 g l^{-1} xylose. The xylose consumption rate was more than twice as fast in YP

Table 1 The genotype and sources of the strains and plasmids used in this study

Strains or plasmids	Genotype	Source
Strains:		
S. cerevisiae H158 (GPY55-15Bα)	α *leu2-3, 112 ura3-52 his4–519 trp1-289 prb1 cir+*	Gregg Payne, University of California, Berkeley
E. coli JM101	r_k' m_k' *supE thi* D(*lac-proAB*) /F′ *traD36 proAB lacI*^{q}*Z* M15	Sambrook et al. (1989)
Plasmids:		
pY6	*bla URA3 ADH1*$_P$*-XYL1-ADH1*$_T$ (←)[a] *PGK1*$_P$*-XYL2-PGK1*$_T$ (→)	Walfridsson et al. (1997)
pDF1	*bla fur1::LEU2*	La Grange et al. (1996)

[a] The direction of the promoter-gene fragment in pY6 is indicated

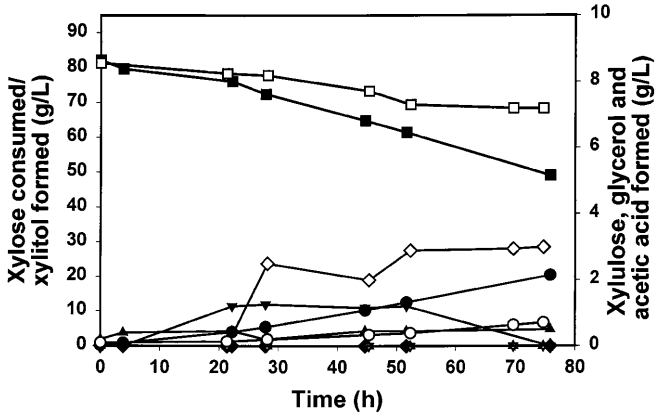

Fig. 1 Xylose utilisation by *S. cerevisiae* [*fur1*: : *LEU2* pY6] in rich (*filled symbols*) and defined medium (*open symbols*). Sugars consumed and products formed are: xylose (■, □), xylulose (◆, ◇), xylitol (●, ○), glycerol (▲, △) and acetic acid (▼, ▽)

medium (0.41 g l^{-1} h^{-1}) than in defined medium (0.15 g l^{-1} h^{-1}). Of the 39% xylose consumed in YP medium, 26% was converted to xylitol and the remainder (13%) assimilated through the pentose phosphate pathway and glycolysis. Only 15% of the xylose was consumed in the defined medium, of which 8% and 4% were converted to xylitol and xylulose, respectively. A very small amount (3%) of the xylose was assimilated in the defined medium. These results clearly demonstrated the limiting effect of defined medium not only on the efficient consumption of xylose but also its assimilation by recombinant *S. cerevisiae* strains. In contrast, fermentations of *S. cerevisiae* H158[*fur1::LEU2* pY6] in complex medium not only showed a twofold higher consumption of xylose compared to defined medium, but also a substantial higher assimilation of xylose in accordance with results obtained with other recombinant xylose-utilising *Saccharomyces* strains (Ho et al. 1998).

Xylose utilisation in the presence of glucose or raffinose as co-substrate

Fermentations of *S. cerevisiae* H158[*fur1*::*LEU2* pY6] were performed for 82 h in YP medium containing 50 g l^{-1} xylose and 20 g l^{-1} glucose or raffinose as co-substrate (Fig. 2A, B). The xylose consumption rate in the presence of a co-substrate was more than threefold higher (1.32 g l^{-1} h^{-1} and 1.46 g l^{-1} h^{-1} for glucose and raffinose, respectively) than when xylose was used alone (0.44 g l^{-1} h^{-1}). The high conversion rate for xylose to xylitol during the first 22 h of fermentation could be ascribed to xylose acting as a redox sink for the reoxidation of NAD^+ necessary for glycolysis under anaerobic conditions (Thestrup and Hahn-Hägerdal 1995; Meinander et al. 1996). However, after exhaustion of the co-substrate, the xylose-consumption rate tapered off to 0.19 g l^{-1} h^{-1} and 0.29 g l^{-1} h^{-1} for glucose and raffinose, respectively. The overall xylose consumption after 82 h was higher when

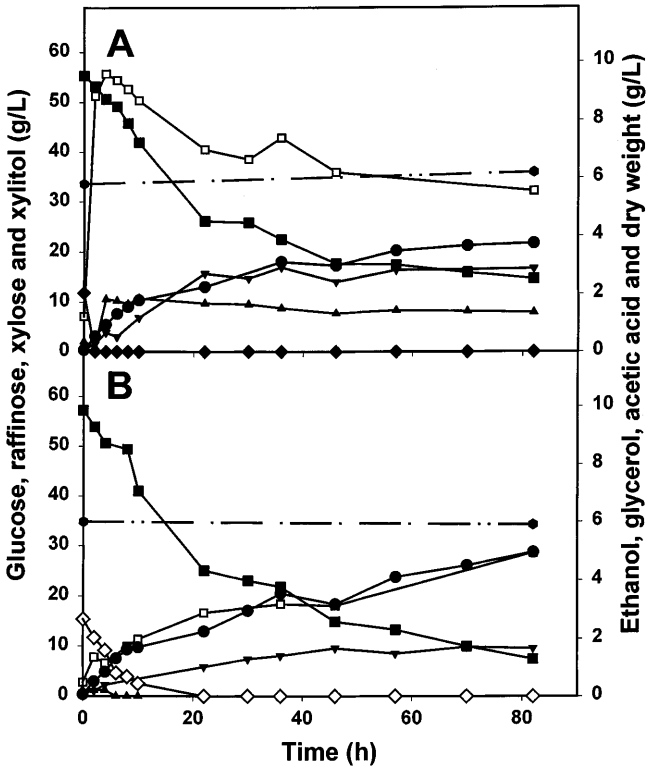

Fig. 2 Xylose utilisation by *S. cerevisiae* [*fur1*::*LEU2* pY6] in the presence of glucose or raffinose as co-substrates in rich medium. Sugars consumed and products formed are: xylose (■), glucose (◆), raffinose (◇), xylitol (●), glycerol (▲), acetic acid (▼), ethanol (□), and dry mass (⬢)

raffinose (85%) rather than glucose (71%) was used as co-substrate. Xylitol production was accordingly higher for raffinose (57%), compared to glucose (43%). The assimilation of xylose through the pentose phosphate pathway was the same for glucose and raffinose, 27%.

A striking difference between the two fermentations was the fast assimilation of glucose (within 4 h) compared to the more gradual assimilation of raffinose (22 h). This resulted in a much higher fermentation rate for glucose as co-substrate as noted with the high ethanol concentration (9.6 g l^{-1} for glucose versus 1.5 g l^{-1} for raffinose) during the first 4 h of fermentation. However, ethanol was consumed again during the rest of the fermentation, yielding 5.5 g l^{-1} at 82 h. These results suggested that ethanol was primarily produced from glucose as co-substrate and not from xylose. However, during the fermentation with raffinose as co-substrate, the ethanol concentration gradually increased from 1.5 g l^{-1} at 4 h to 4.9 g l^{-1} at 82 h. Considering that only 7.1 g l^{-1} fructose was released from the 20 g l^{-1} raffinose, part of the xylose had to be converted to ethanol. Furthermore, the high fermentation rate with glucose as co-substrate yielded moderate levels of glycerol and acetic acid, whereas raffinose yielded no glycerol and much less acetic acid (Table 2).

Raffinose has several advantages over glucose as co-substrate to study xylose metabolism in yeast. The slow

Table 2 Product formation by *S. cerevisiae* after about 80 h fermentation in defined and complex medium with xylose, with or without either glucose or raffinose, as substrate

Product	Fermentation medium			
	defined (xylose, 80 g l^{-1})	complex (xylose, 80 g l^{-1})	complex (xylose, 50 g l^{-1}; glucose, 20 g l^{-1})	Complex (xylose, 50 g l^{-1}; raffinose, 20 g l^{-1})
Substrate remaining	–	–	–	–
Glucose	–	–	0.0	–
Raffinose	–	–	–	0.0
Xylose	68.3	48.9	14.6	7.5
Products formed				
Xylulose	3.0	0.0	0.0	0.0
Xylitol	6.7	20.4	21.7	28.7
Glycerol	0.0	0.5	1.4	0.0
Acetic acid	0.0	0.0	2.9	0.0
Ethanol	0.0	0.0	5.5	4.9

release and simultaneous utilisation of fructose by the yeast cells avoid the induction of strong glucose repression during the first phase of the fermentation (Gancedo 1998). In addition, the production of by-products other than ethanol, such as glycerol and acetic acid, as a result of high fermentation rates are limited. It is also tempting to speculate that the induction of high affinity hexose transporters in the absence of glucose (Reifenberger et al. 1997) allowed more efficient transport of xylose into the yeast cell. Raffinose thus may provide an alternative physiological background to study xylose consumption, assimilation and conversion to ethanol by yeast.

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